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Effects of glycerol on *Pseudomonas fluorescens* BTP1 freeze-dried

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Abstract

The storage stability of freeze-dried powders was studied by parameters such as loss of viability on the Plate Count Agar (PCA). Powder with glycerol (PG) contains $8.4x10^{10}$cfu/g before storage $1.1x10^{10}$cfug after 3 months at 4°C and $6.0x10^{8}$cfu/g after 3 months at 20°C. The concentration of soluble proteins (mg/g) decrease during storage at 4°C from 3.77 to 0.80 after 90 days; and the ratios of unsaturated to saturated fatty acids (C18:3/C16:0 and C18:2/C16:0) decrease respectively from 0.05 to 0.04 and 0.007 to 0.004 after 3 months at 4°C. This ratio characterises the membrane fluidity. Powder without glycerol (PS) contains $1.1x10^{10}$ cfu/g before storage and 1.4×10^{8} cfu/g after 3 months at 4°C and 1.4×10^{7} cfu/g after 3 months at 20°C. The concentration of soluble proteins (mg/g) decrease during storage at 4°C from 4.08 to 0.42 after 90 days, the glutathione concentration decrease during storage at 4°C from 2.2 to 1.4. The beneficial effect of glycerol on fatty acid composition during freeze-drying is shown and the ratios of unsaturated to saturated fatty acids (C18:2/C16:0 and C18:3/C16:0) decrease respectively from 0.019 to 0.004 and 0.054 to 0.036 after 90 days storage at 4°C. Analysis by flow cytometry was used to assess the physiological state in which cells are at the end of freeze-drying. We found 13.5% live cells, 36.1% dead cells and 50.4% cells in

an intermediate state for powder with glycerol (PG) after freeze-drying. These results shows that glycerol play an important role in *Pseudomonas fluorescens* BTP1 desiccation during freeze-drying, by maintaining a degree of viability after freeze-drying and during storage.

Keywords: Bio-fungicide, cellular fatty acids, flow cytometry, glutathione, protein.

ABBREVIATIONS

cFDA, carboxyfluoresceindiacetate;
Cfu, colony forming unit;
CG, Cream with 2% glycerol;
CS, Cream without glycerol;
DNTB, 5,5'-dithiobis(2-nitrobenzoic acid) ;
EDTA, Ethylenediaminetetraacetic acid;
FAME, fatty acid methyl esters;
GSH, Glutathione;
NADP, Nicotinamide adenine dinucleotide phosphate;
PCA, plate count agar;
PG, powder with 2% glycerol;
PI, propidium iodide;
PS, powder without protective compound;
PUFA, polyunsaturated fatty acid;
ROS, Reactive Oxygen Species;
TNB, 2-nitro-5 thiobenzoic acid

Introduction

Some fluorescent pseudomonads are referred to as plant growth-promoting rhizobacteria (PGPR) and their effectiveness in controlling a number of plant diseases caused by soil-borne pathogens has been widely documented (Bakker et al., 2007; Ongena et al., 1999). *Pseudomonas spp*. can colonise the roots of crop plants and produce antifungal metabolites represent a real alternative to the application of chemical fungicides (Walsh et al., 2001). *Pseudomonas fluorescens* is interesting in agriculture and horticulture biocontrol as fungicide. For this use, powder is generally produced to assure facilities and longer life of the product. Freeze-drying is most convenient, commonly and successful method of preserving bacteria (Liu et al., 2009; Walsh et al., 2001).

Freeze-drying is a technique suitable for production of concentrated bacterial cultures with the advantage that the dried material can be stored at ambient temperature and easily transported (Coulibaly et al., 2009; Palmfeldt et al., 2003). But, this technique brings about undesirable side effects, such as modifications in the physical state of membrane lipids and proteins altered, causing the loss of cell viability during the process as well as during subsequent storage (Garait et al., 2005;

Nanasombat and Sriwong, 2007). Lipid oxidation of membrane cells was deemed responsible for cell death during freeze-drying and during storage (Teixeira et al., 1996). In our previous study, we had shown lipids oxidation by decrease of polyunsaturated fatty acids (C18:2 and C18:3) and the importance of protective compounds to minimize loss of cell viability. Those data support further the view that polyunsaturated fatty acids (linoleic and linolenic acids) play a key role in determining cellular membrane susceptibility to oxidative or heat stress and glycerol has been the best protective compounds (Mputu Kanyinda et al., 2012).

This oxidation was often due to the reactive oxygen species (ROS). This ROS can damage proteins, modify bases and sugars in deoxyribonucleic acid (DNA) (Blair, 2008). In the dry state bio-molecules become more susceptible to the attack of oxygen (Garait et al., 2005). It is reported that the presence of antioxidants increased the survival rate of dried bacteria during storage (Carvalho et al., 2003; Coulibaly et al., 2009). Most cells possess several key mechanisms to limit their exposure to ROS. One of the major components of cellular antioxidant defenses is the thiol compound glutathione. When cells are exposed to oxidant species such as H_2O_2, O_2, reduced glutathione (GSH) is oxidized to a dimer oxidized glutathione, or glutathione disulfide (O'Malley et al., 2004; Richard et al., 1997). In addition to its key role in maintaining the proper oxidation state of protein thiols in bacteria, glutathione also serves a key function in protecting the cell from the action of low pH, chlorine compounds, and oxidative and osmotic stresses (Masip et al., 2006). The consumption of glutathione during storage could inquire us about the oxidation phenomena. The viability of cultures is traditionally quantified by the plate count technique, which tests the ability of the cells to reproduce on an appropriate medium. Although colony counting is the conventional method for quantitative survival studies, this technique has three major drawbacks.

Firstly it requires a long incubation time (24–72 h), depending on the bacterial species), secondly it often leads to an underestimation of viable cell counts as a result of cell clumping and chain formation and thirdly it only takes the bacteria that replicate under the conditions provided for growth into account. As a consequence, some cells, which are known as viable but non-cultivable, are not counted by this method, just like the cells that are dead, sublethally damaged, injured, inhibited, dormant or inactive. Nevertheless, they may return to a physiologically active state when suitable conditions are subsequently restored (Kaprelyants et al., 1999; Rault et al., 2007). Alternative techniques for viability assessment are desired for fundamental as well as routine microbiology research, although they have to be rapid and reliable. Flow cytometry (FCM) is an appealing technique for fast viability assessment (Bunthof and Abee, 2002; Bunthof et al., 2001a; Gasol and Del Giorgio, 2000). The first objective of the present study was to evaluate impact of freeze-drying and glycerol effect on *Pseudomonas fluorescens* BTP1 by using whole-cell fatty acid methyl ester (FAME) and flow cytometry analysis; the second objective was to evaluate the cell oxidation by glutathione and protein level during storage.

Materials and methods

Organisms and cultivation

The strain used in our study is *Pseudomonas fluorescens* BTP1 of Wallon Center of Industrial Biology laboratory (CWBI). The strain was grown in 100 L bioreactor (Biolafitte) containing 60 liters of 863 medium, for 20 hours and then concentrated 20 times by centrifugation (Sorval RC 12 BP) at 4700 rpm. After that, pellets were divided in two parts, one with protecting compound: 2% w/w glycerol and one without (Coulibaly et al., 2009; Mputu Kanyinda et al., 2012) and freeze-dried in a freeze-drier (LOUW KOELTECHNIEK BVBA) with a standard program by increasing the temperature gradually from -25°C to 25°C at 0.9 mbar pressure during 48h (Jørgensen et al., 1994; Yao et al., 2010).

Measurement of cultivability

Cultivability of cells was evaluated by Plate Counts Agar. After serial dilutions in peptone water, cells were plated onto solid 868 agar and incubated at 30°C for 24 h. Each result was the geometrical mean of at least three counts. Each result was the geometrical mean of at least three counts (Coulibaly et al., 2009).

Fluorescent probes and staining protocols for flow cytometry analysis

Carboxyfluoresceindiacetate (cFDA) was used to assess *Pseudomonas Fluorescens* BTP1 viability, whereas the nucleic acid dye, propidium iodide (PI), made it possible to quantify damaged and dead cells. Before staining, cell suspensions of *Pseudomonas fluorescens* BTP1 were diluted in peptone water at 0.2 optic density to 590 nm, centrifuged 2 minutes at 12000 rpm, and the pellets were resuspended in 1ml PBS buffer to reach approximately 10^6 cells/ml. One milliliter of the diluted suspension was supplemented with 10 µL of cFDA and incubated for 15 min at 37°C for viability assessment, or with 10 µl of PI and incubated for 30 min at 37°C for membrane integrity assessment (Bunthof et al., 2001a; Rault et al., 2007). Live/dead assays were done by dual staining of each sample to differentiate viable, dead and stressed cells. The same dye concentrations were used. The diluted suspension was first incubated with 10 µL of PI and 10 µl of cFDA for 15 min. After centrifugation (Fischer BioblockScientifik) and rinsing three times at 12000 rpm for 1 minute in 1 ml of PBS buffer, cell pellets were resuspended in 1 ml of PBS buffer before analysis by flow cytometry(Bunthof et al., 2001a; Rault et al., 2007).

The subpopulations were identified using dot plots. Gates were defined in the dot plots of FSC, SSC, green fluorescence and red fluorescence, thus allowing the software to separate the different events. Before analysis, detectors were adjusted and compensation setting was performed on a sample with unstained cells. The corresponding signal on the dot plots was set in the lower left quadrant in order to eliminate cellular auto-fluorescence. Data were analyzed with the aid of statistical tables, which indicated the percentages of stained cells determined by each detector (Miao et al., 2009; Rault et al., 2007).

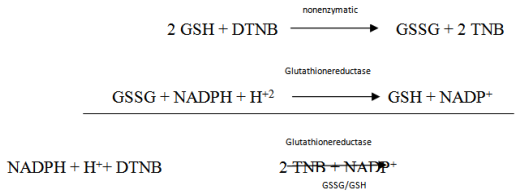
Fatty acids analysis by gas-liquid chromatography

Total lipids were extracted overnight from dried cells (1g) with chloroform-methanol

(2:1 v/v) mixture. Saline solution NaCl 0.88% (w/v) is added in order to obtain 2 phases shakes overnight Folch method modified (Folch et al., 1957). The lower organic phase contains all lipids excepted gangliosides. Chloroform-methanol extracts were pooled, filtered, and then evaporated and concentrated under reduced pressure at 35°C. Fatty acid methyl esters (FAME) were prepared from the concentrate with 14% (w/w) solution of boron trifluoride (BF_3) in methanol as reagent (Sigma, St Louis, MO, USA). After heating at 70 °C in a water bath for 90 min, 0.5 ml of saturated NaCl, 0.2 ml of sulphuric acid (10%) and 0.5 ml of n hexane were added. The methylated fatty acids were taken from the upper phase after decanting. Gas chromatographic analysis of the methyl esters was carried out on a HP 6890 (Hewlett Packard) gas chromatograph equipped with a flame ionization detector at 250 °C (Coulibaly et al., 2009; Mputu Kanyinda et al., 2012). A capillary column (30x0.25 mm) was used. Helium was used as carrier gas (2.4 ml/min) and the injection volume was 1 µl. Injection was done at 250 °C in splitless mode for 1 min. The oven temperature was held at 50 °C for 1 min, increased by 30 °C/min to 150 °C, and then from 150 °C to 240 °C at 4 °C/min with a final hold of 10 min at 240 °C. Fatty acids methyl esters were identified by comparing their retention times with standard mixtures FAME MIX 47885U (Supelco, Bellefonte, USA). The relative fatty acid content was estimated as a percentage of the total peak area using a DP 700 integrator. The relative content (%) of palmitic (C16:0) acid for two reasons: first C16:0 had the highest proportion at most points relative to other fatty acids and second C16:0 did not change significantly during storage.

Determination of cellular glutathione (GSH)

1 g of powder was washed at least 10^8 cells with PBS, then suspend to a cell density of 10^8 cells per ml in PBS and transfer the cells to a micro-centrifuge tube (Fischer BioblockScientifik). Cells were centrifuged at 600 x g to obtain a packed cell pellet, the supernatant was removed and the volume of the pellet was measured, 3 volumes of the 5% SSA solution were added to the packed cell pellet and vortexed. Freeze and thaw the suspension twice (use liquid nitrogen or a freezer at - 80 °C to freeze and a 37 °C bath to thaw) and leave for 5 minutes at 2–8 °C. Centrifuge the extract at 10,000 x g for 10 minutes and measure the volume of the supernatant and use this as the original sample volume in the calculation for glutathione determination. Keep the sample at 2–8 °C. For the assay procedure, there may be a need for sample dilution (up to 20-fold) in order to stay in the detection range. The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present will also react to give a positive value in this reaction (Fahey et al., 1978; Nair et al., 1991; Smirnova and Oktyabrsky, 2005; Wright and Viola, 1998).



The reaction rate is proportional to the concentration of glutathione up to 2 mM. The yellow product, 5-thio-2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample.

Proteinsdetermination

1 g of *Pseudomonas fluorescens* BTP1 powder was washed twice by centrifugation (14000g, 30 minutes at 4°C) in 10 ml of Tris 50mM, pH 8.8. The cells were then lysed 3 minutes by sonication (BandelinSonoplus HD 2070) in Tris-EDTA (50mM, pH 8.8). After sonication, centrifugation is perfomed (24,000g, 15 minutes at 4°C) to remove cells and cellular debris crushed. The supernatant containing soluble proteins was assayed by Bicinchoninic Acid Protein Assay kit from Sigma (Brown et al., 1989; Chart, 1995).

Statistical analysis

Data from three replications were analyzed by using analysis of variance to determine if significant difference ($P \leq 0.05$) existed between mean values.

Results and discussion

Table1 shows the six main fatty acids recovered from cell pellets with and without glycerol before and after freeze-drying and fig.1 shows the survival Cfu/g of freeze-drying *Pseudomonas fluorescens* BTP1 strain during 90 days storage at 4 and 20°C. Figure 2 shows the evolution of soluble proteins concentration level of *Pseudomonas fluorescens* BTP1 during storage at 4 and 20°C and fig. 3 shows the ratios of polyunsaturated fatty acid by saturated fatty acid (C18:3 and C18:2 by C16:0) of *Pseudomonas fluorescens* BTP1 freeze-dried during storage at 4°C and 20°C; this ratio indicate that freeze-drying and storage induces lesions on membrane lipids. Figure 4 shows the consumption of glutathione (GSH) in *Pseudomonas fluorescens* BTP1 during storage at 4 and 20°C, this consumption shows that we have an oxidation phenomenon of glutathione. Figure 5 shows the physiological state of cell (*Pseudomonas fluorescens* BTP1 pellet and powder) after PI/cFDA double staining by Flow cytometry.

After freeze-drying, powders with and without glycerol were sealed in aluminium bags under vacuum and stored at 4 or at 20°C. The storage stability of freeze-dried powders was studied using parameters such as viability by using a Plate Count Agar (PCA). Powder without glycerol (PS) contains 1.1×10^{10} cfu/g before storage; after 90 days storage we have 1.4×10^{8} cfu/g at 4°C and 1.4×10^{7} cfu/g at 20°C.We have a decrease of a factor 100 for powder stored at 4°C and a decrease of a factor 1000 for the powder stored for 20°C. Loss of viability is more pronounced at 20°C than at 4°C. Powder with glycerol (PG) contains 8.4×10^{10}cfu/g before storage and after 90 days storage we have 1.1×10^{10}cfu/g at 4°C and 6×10^{8}cfu/g at 20°C.

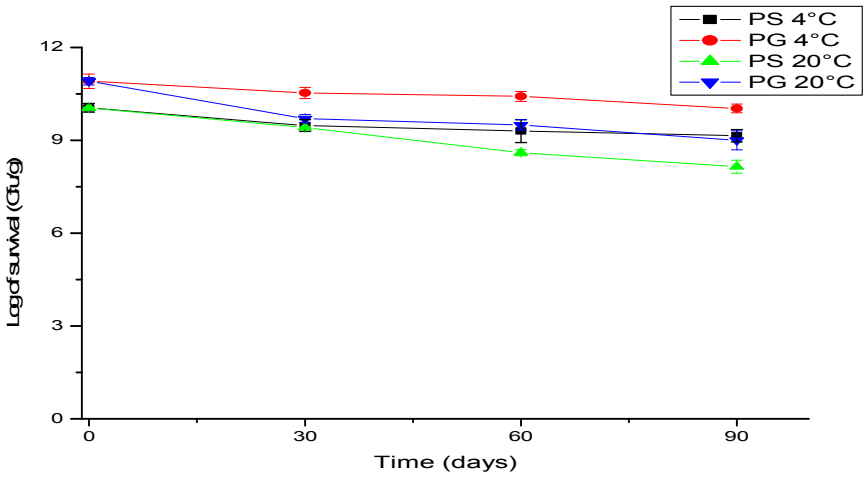


Figure 1. Survival Cfu/g of freeze-dried *Pseudomonas fluorescens* BTP1 during 90 days storage at 4°C and 20°C.

We have a decrease of a factor 10 for powder stored at 4°C and a decrease of a factor100 for the powder stored at 20°C. It was found that, regardless of packaging mode or storage temperatures, the viability of the freeze-dried decreased as the storage time increased. As predictable the powder with glycerol has better viability than the powder without glycerol. We observe a decrease of protein level during storage, the concentration of soluble protein (mg/g) decreases during storage. For powder without glycerol we have 4.08 mg/g of powder before storage and after 90 days storage we have 0.42 mg/g of powder at 4°C and 0.27 mg/g of powder at 20°C. We have for the powder with glycerol 3.77 mg/g of powder before storage and after storage 0.80 mg/g of powder at 4°C and 0.61mg/g of powder at 20°C.

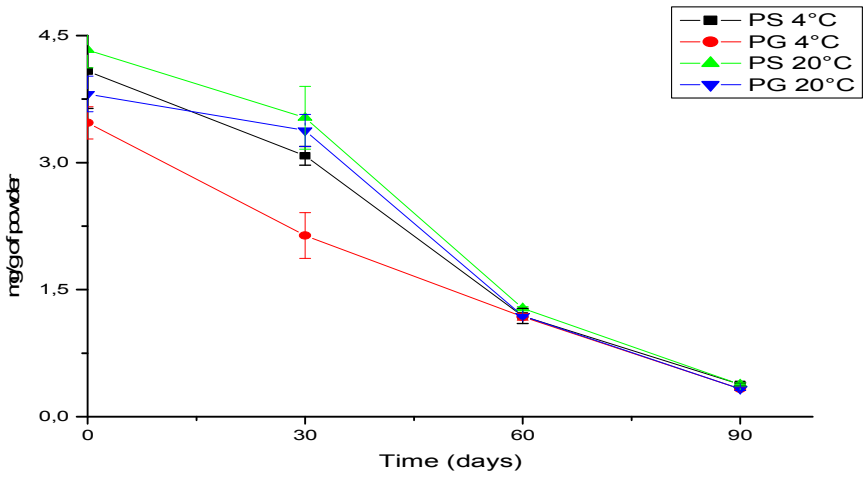


Figure 2. Evolution of the concentration of soluble protein during storage at 4 and 20°C of *Pseudomonas fluorescens* BTP1 powder.

This decrease in proteins concentration is function of time and storage temperature. We observe a great decrease at 20°C than at 4°C, but it is more pronounced for the powder without glycerol than the powder with glycerol. The decrease of proteins concentration shows that proteins are altered and insolubilized during storage and proteins are more altered in the powder without glycerol than the powder with glycerol.

The pellets with and without glycerol were analysed for fatty acid determination before and after freeze-drying, results are reported in table 1. The fatty acid composition of *Pseudomonas fluorescens* BTP1 was characterized in order to better understand the damage suffered by the cell membrane during freeze-drying and storage. This result shows the relative percentage of fatty acid before and after freeze-drying. We have six main fatty acids in *Pseudomonas fluorescens* BTP1 cellular membrane. Palmitic acid (C16:0) is the majority fatty acid in *Pseudomonas fluorescens* BTP1, the addition of glycerol before freeze-drying allows to maintain fatty acids C18:2 and C18:3 (Mputu Kanyinda et al., 2012).

Table1. Cellular fatty acid composition of *Pseudomonas fluorescens* BTP1 before and after freeze-drying

Treatment		Relative content (%)					
		C16 :0	C16 :1	C18 :0	C18 :1	C18 :2	C18 :3
Before freeze-drying	CS	54,4±0,2	16,1±0,3	4,1±0,1	21,1±0,2	1,7±0,1	2,6±0,1
	CG	55,0±0,3	17,6±0,2	4,1±0,2	19,1±1,0	1,6±0,3	2,6±0,2
After freeze-drying	CS	53,7±0,1	17,1±0,2	4,6±0,1	22,4±1,0	0,9±0,2	1,3±0,3
	CG	54,8±1,3	19,3±0,0	3,5±0,2	19,0±0,1	1,0±0,2	2,4±0,2

The six main fatty acids are palmitic (C16:0), pamitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3).

Membrane lipids play an important role in cytoplasmic membrane permeability, it seemed probable that the damage of membrane during freeze-drying and during storage were caused by changes in lipid profile (Teixeira et al., 1996). Figure 3shows the relation between changes in the unsaturated/saturated fatty acids and storage time.

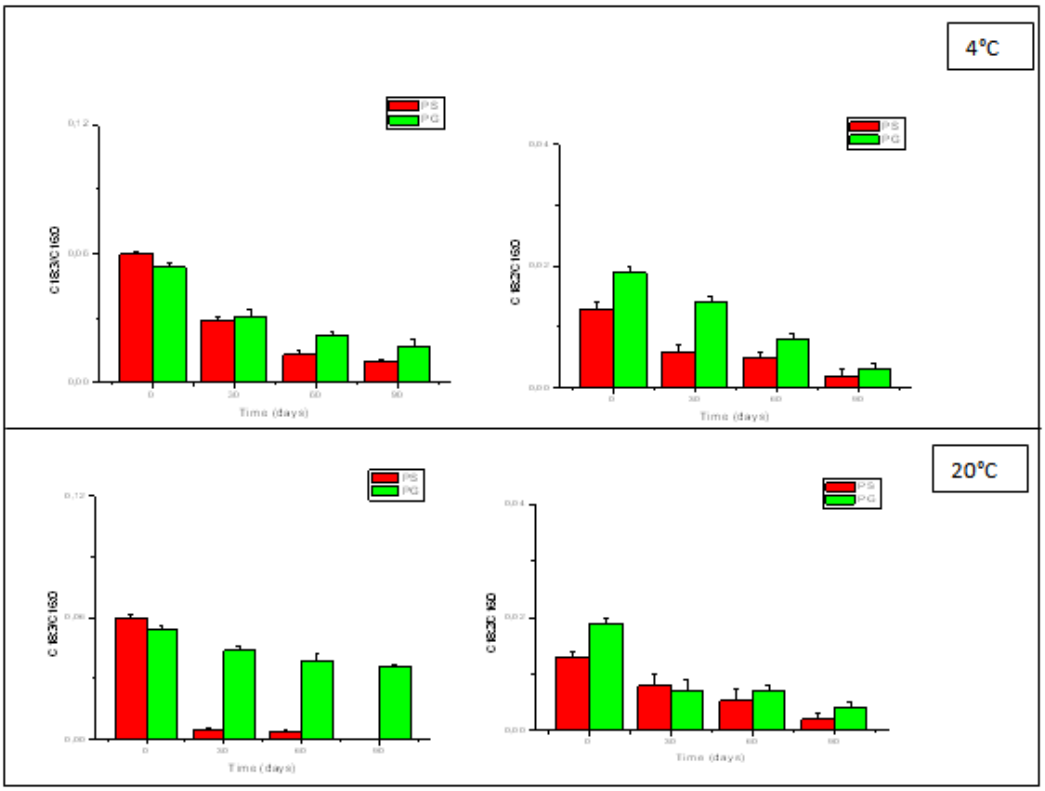


Figure 3: C18:2/C16:0 and C18:3/C16:0 ratios of *Pseudomonas fluorescens* BTP1 powders during 90 days storage at 4°C or 20°C in vacuum-sealed aluminium foil.

The ratios of unsaturated to saturated fatty acids (C18:2/C16:0 and C18:3/C16:0) for powder without glycerol at 4°C decrease respectively from 0.013 before storage to 0.002 after 90 days storage and 0.06 before storage to 0.001 after 90 days. At 20°C we have a decrease from 0.013 before storage to 0.001after 90 days storage and 0.06 before storage to 0.0 after 90 days storage. This ratio indicates that freeze-drying and storage induces lesions in the cellular lipids (Coulibaly et al., 2009). These ratios (C18:2/C16:0 and C18:3/C16:0) for the powder with glycerol at 4°C decrease respectively from 0.019 before storage to 0.004 after 90 days storage and 0.054 before storage to 0.036 after 90 days storage. At 20°C we have a decrease for the C18:2/C16:0 ratio from 0.019 before storage to 0.003 after 90 days storage and from 0.054 before storage to 0.017 after 90 days storage for the C18:3/C16:0 ratio. Comparing the ratios C18:2 and C18:3 by C16:0 of the powder with and without glycerol, it was observed that powder without glycerol is more affected by storage than cells with glycerol.

Comparatively to the oxidation of lipids, glutathione are also oxidized. Glutathione was measured before and during storage. Glutathione (GSH) is the major cellular thiol participating in cellular redox reactions and thioether formation. Glutathione is a protective compound naturally produced by the cell; GSH is known

to regulate many of the important functions of cell. The concentration of glutathione decrease during storage respectively at 4°C and 20°C for powder without glycerol; from 2.2 µmol/ g of powder before storage to 1.4 µmol/ g of powder and 0.6 µmol/ g of powder after 90 days storage. And for the powder with glycerol, we have a decrease from 2.2 µmol/ g powder before storage to 1.7 µmol/ g powder after 90 days storage at 4°C and a decrease from 2.2 µmol/ g powder before storage to 0.6 µmol/ g powder after 90 days storage at 20°C. This decrease of glutathione concentration is more pronounced for powder without glycerol than powder with glycerol, the fact that the concentration is lower at 20 ° C shows that the oxidation phenomena are marked at high storage temperature. Consumption of glutathione is evidence that some of the phenomena involved in *Pseudomonas fluorescens* BTP1 death are oxidation phenomena.

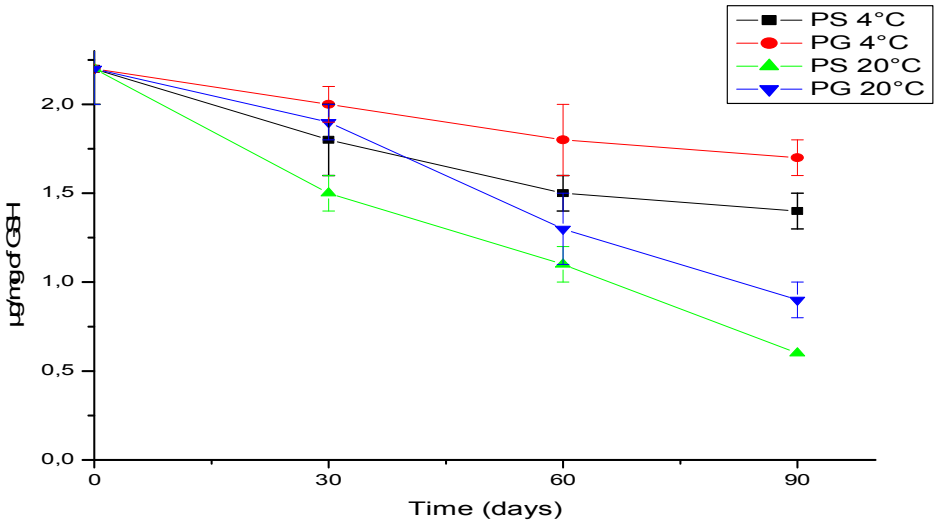


Figure 4: Concentration of glutathione (GSH) in *Pseudomonas fluorescens* BTP1freeze-drying during storage at 4 and 20°C.

Analysis by flow cytometry was used to assess the physiological state in which cells are at the end of freeze-drying. This technique allows the determination of relative percentages of viable cells, dead cells and intermediate cells (viable but non-cultivable) after double staining by the PI and cFDA(Miao et al., 2009). Figure 5 shows that for the cream before freeze-drying (control) we have 1.4% of dead cells, 4% of intermediate cells and 94.5% live cells, for powder without glycerol we have 44.9% of dead cells, 47.2% of intermediate cells and 7.9% of live cells and for powder with glycerol we have 36.1% of dead cells, 50.4% cells and 13.5% of live cells. We found in our previous results that majority of cells are in an intermediate state after 270 days storage at 20°C (Mputu Kanyinda et al., 2012).

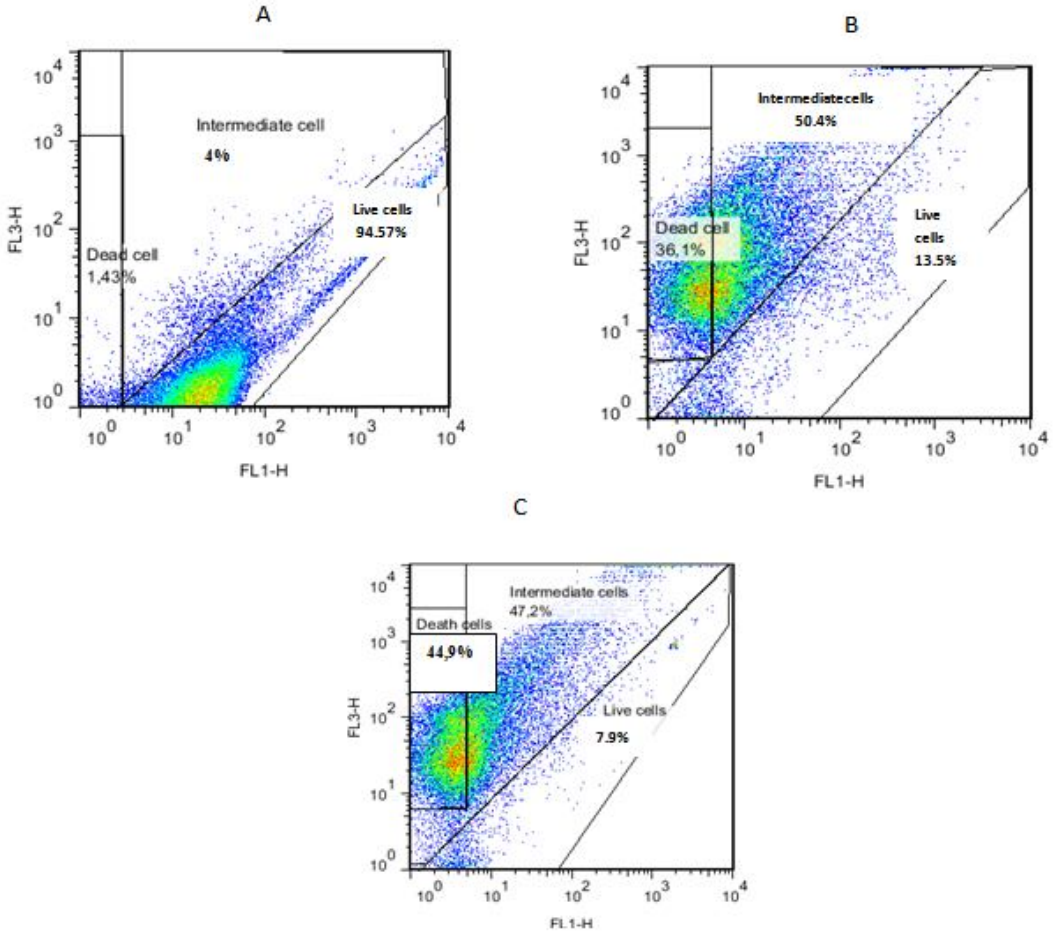


Figure 5. Flow cytometry analyzed of *Pseudomonas fluorescens* BTP1 after PI/cFDA double staining (A.Cream of cells before freeze-drying, B. Powder with 2% glycerol, C. Powder without glycerol).

Comparatively with our present results (Mputu Kanyinda et al., 2012), we observe that cells are more affected during freeze-drying than during storage and the best results are obtained by powder with glycerol. By comparing the results obtained from different powders with cream, we note that the freeze-drying affects considerably the cells.

Conclusion

The results presented here provide experimental support to the hypothesis that *Pseudomonas fluorescens* BTP1 is sensitive to freeze-drying, because gram negative not well adapted to desiccation by comparing with gram positive. Low viability during storage was associated with a decrease in C18:2/C16:0 and C18:3/C16:0 ratios, more pronounced at 20°C.

Glutathione consumption confirms its oxidation during storage and decrease of soluble protein concentration shows that proteins are altered during storage. These data further support the view that polyunsaturated fatty acids (linoleic and linolenic acids) and GSH play a key role in determining cellular susceptibility to oxidative. The beneficial effect to use glycerol is demonstrated by better viability, a low decrease of the ratios of unsaturated to saturated fatty acids (C18:3/C16:0 and C18:2/C16:0), a lower decrease of GSH during storage and a best percentage of living cells by flow cytometry after freeze-drying. The decrease in the ratio (C18:3/C16:0 and C18:2/C16:0) fatty acids, the consumption of glutathione and the decrease of soluble proteins proves that oxidation phenomenon are responsible for the mortality of *Pseudomonas fluorescens* BTP1 during storage. The use of glycerol during freeze-drying step has resulted in improved viability. This shows the beneficial effect to use glycerol in the conservation of *Pseudomonas fluorescens* BTP1.

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